

TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371

044481-5043

U.S. Application No.

097673302

International Application. No.	International Filing Date	Priority Date Claimed
<b>PCT/US99/08285</b>	<b>15 April 1999</b>	<b>15 April 1998</b>

Title of Invention

**TRANSGENIC MAMMALS EXPRESSING MUTANT GP IIIa**

Applicants For DO/EO/US

**Deborah Ann LAW and David R. PHILLIPS**

Applicants herewith submit to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☒ has been transmitted by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventors (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.  
☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☒ A Verified Statement Claiming Small Entity Status
16. ☒ Other items or information:
  - a. ☒ Copy on International Preliminary Examination Report dated 29 January 1997
  - b. ☒ WIPO Publication No. WO 99/53032 of International Application No. PCT/US99/08285 including International Search Report
  - c. ☒ Copy of PCT/IB/308

09/673302

PCT/US99/08285

044481-5043

17. [X] The following fees are submitted:

**Basic National Fee (37 CFR 1.492(a)(1)-(5)):**

Search Report has been prepared by the EPO or JPO.....\$860.00  
 International preliminary examination fee paid to  
 USPTO (37 CFR 1.482).....\$690.00  
 No international preliminary examination fee paid to  
 USPTO (37 CFR 1.482) but international search fee  
 paid to USPTO (37 CFR 1.445(a)(2)).....\$710.00  
 Neither international preliminary examination fee  
 (37 CFR 1.482) nor international search fee  
 (37 CFR 1.445(a)(2)) paid to USPTO.....\$1000.00  
 International preliminary examination fee paid to USPTO  
 (37 CFR 1.482) and all claims satisfied provisions  
 of PCT Article 33(2)-(4).....\$100.00

**ENTER APPROPRIATE BASIC FEE AMOUNT =** \$860.00

Surcharge of \$130.00 for furnishing the oath or declaration later than

[ ] 20 [ ] 30 months from the earliest claimed priority date

(37 CFR 1.492(e)).

\$

Claims	Number Filed	Number Extra	Rate	
Total Claims	68- 20 =	48	X \$18.00	\$864.00
Independent Claims	5 - 3 =	2	X \$80.00	\$160.00
Multiple dependent claim(s) (if applicable)			+ \$270.00	\$

**TOTAL OF ABOVE CALCULATIONS =** \$1884.00

Reduction by 1/2 for filing by small entity, if applicable. Verified

Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28)

-\$942.00

**SUBTOTAL =** \$942.00

Processing fee of \$130.00 for furnishing the English translation later

than [ ] 20 [ ] 30 months from the earliest claimed priority date

(37 CFR 1.492(f)).

+\$

**TOTAL NATIONAL FEE =** \$942.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The

assignment must be accompanied by an appropriate cover sheet

(37 CFR 3.28, 3.31).

\$40.00 per property

+\$

**TOTAL FEES ENCLOSED =** \$942.00

Amount to be

refunded

\$

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- a. [ ] A check in the amount of \$ to cover the above fees is enclosed.
- b. [X] Please charge my Deposit Account No. 50-0310 in the amount of **\$942.00** to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. [ ] Except for issue fees payable under 37 C.F.R. §1.18, the Commissioner is hereby authorized by this paper to charge any additional fees during the entire pendency of this application including fees due under 37 CFR §1.16 and §1.17 which may be required, or credit any overpayment to Deposit Account No. 3-4520.

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Submitted: **October 16, 2000**

PATENT  
Attorney Docket No. : 44481-5043-PR

Applicants: Deborah Ann LAW et al.  
U.S. Serial No.: 09/060,051  
Filed: April 15, 1998  
For: TRANSGENIC MAMMALS EXPRESSING MUTANT GP IIIa

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY  
STATUS (37 CFR 1.9(f) and 1.27(c))--SMALL BUSINESS CONCERN**

I hereby declare that I am

- ☐ the owner of the small business concern identified below:  
☒ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN: COR THERAPEUTICS

ADDRESS OF CONCERN: 256 E. Grand Avenue, South San Francisco, California 94080

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, in the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third-party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed, so and remain with the small business concern identified above with regard to the invention, entitled:

**TRANSGENIC MAMMALS EXPRESSING MUTANT GP IIIa**

by inventors: Deborah Ann LAW and David R. PHILLIPS

described in

- ☐ the specification filed herewith.  
☒ application serial no. 09/060,051 filed April 15, 1998  
☐ patent no.          issued

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights in the invention is listed below and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

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**TRANSGENIC MAMMALS EXPRESSING MUTANT GP IIIa****RELATED APPLICATIONS**

This application is related to U.S. Patent Application No. 08/734,607, filed October 18, 1996; U.S. Provisional Application No. 60/031,665, filed November 21, 1996; U.S. Provisional Application No. 60/042,093, filed March 28, 1997; and, U.S. Patent Application No. 08/975,653, filed November 21, 1997; all of which are hereby incorporated by reference in their entirety.

**FIELD OF THE INVENTION**

The present invention relates to mammals into which foreign DNA has been introduced or in which certain codons of integrin-encoding genes have been replaced, thereby generating transgenic or genetically-engineered mammals. In particular, the present invention provides a transgenic mammal in which the endogenous GP IIIa gene (also known as  $\beta 3$ ) has been replaced in whole or in part with a mutant GP IIIa gene in which one or both of the two phosphorylatable cytoplasmic tyrosine residues have been replaced with non-tyrosine residues, for example, such as phenylalanine. Since the cells, platelets in particular, that are found in the blood of the resultant transgenic mammals that express an altered GP IIIa gene cannot undergo tyrosine phosphorylation, either in whole or in part, as occurs in their wild type counterparts, these animals provide a useful tool for assessing the importance of the phosphorylation of these tyrosine residues for platelet function. The present invention is also useful for studying the effect of the mutant GP IIIa integrin subunit on biological processes other than platelet formation.

**BACKGROUND OF THE INVENTION**

All of the publications and patent applications that are identified in this specification are hereby incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

## A. Integrins

Integrins are a family of  $\alpha\beta$  heterodimers that mediate adhesion of cells to extracellular matrix proteins and to other cells (Clark *et al.*, Science (1995) 268:233-239). Integrins also bind to the actin cytoskeleton through a series of intermediate proteins, and thus provide a link between the extracellular matrix and the intracellular cytoskeleton and its associated motile machinery. Such transmembrane linkages are required for cell migration. Many biological responses are dependent at least to some extent upon integrin-mediated adhesion and cell migration, including embryonic development, hemostasis, clot retraction, mitosis, angiogenesis, inflammation, immune response, leukocyte homing and activation, phagocytosis, bone resorption, tumor growth and metastasis, atherosclerosis, restenosis and wound healing.

Members of the integrin family also participate in signal transduction. This is evidenced by an alteration in the adhesive affinity of cell surface integrins in response to cellular activation, termed inside-out signal transduction. Additionally, effects on intracellular signaling pathways following integrin-mediated adhesion have been observed, termed outside-in signal transduction.

The integrin family consists of 15 related known  $\alpha$  subunits ( $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 6$ ,  $\alpha 7$ ,  $\alpha 8$ ,  $\alpha 9$ ,  $\alpha E$ ,  $\alpha V$ ,  $\alpha IIb$ ,  $\alpha L$ ,  $\alpha M$ , and  $\alpha X$ ) and 8 related known  $\beta$  subunits ( $\beta 1$ ,  $\beta 2$ ,  $\beta 3$ ,  $\beta 4$ ,  $\beta 5$ ,  $\beta 6$ ,  $\beta 7$ , and  $\beta 8$ ). Luscinskas *et al.*, *FASEB J.*, 8: 929-938 (1994). Integrin  $\alpha$  and  $\beta$  subunits are known to exist in a variety of pairings as indicated in Figure 1. Integrin ligand specificity is determined by the specific pairing of the  $\alpha$  and  $\beta$  subunits, although some redundancy exists as several of the integrins are known to bind the same ligand.

Two known pairings of the  $\beta 3$  subunit have been observed: with  $\alpha V$  to make  $\alpha V\beta 3$ , the Vitronectin Receptor; and with GP IIb to make GP IIb-IIIa, the Fibrinogen Receptor.  $\alpha V\beta 3$  is widely distributed, is the most promiscuous member of the integrin family and mediates cellular attachment to a wide spectrum of adhesive proteins, mostly at the R-G-D sequence on the adhesive protein. The biological processes mediated by  $\alpha V\beta 3$  are diverse and include bone resorption, angiogenesis, tumor metastasis and restenosis.  $\alpha V\beta 3$  is known to signal upon adhesive protein ligation (P.I. Leavesley, *et al.*, *J. Cell Biol.* 121:163-170 (1993)). As an example, endothelial cells undergo apoptosis when relieved of ligation (P.C. Brooks, *Cell* 79:1157-1164 (1994)).

## B. Interaction of Integrins with Known Cytoskeletal Proteins

The binding of unmodified  $\alpha$  and  $\beta$  subunit cytoplasmic domains of integrins to a variety of cytoskeletal and signaling proteins has been documented. S. Dedhar *et al.*,  
5 *Curr. Opin. Cell Biol.* 8:657-669 (1996). Morphological studies have shown that many of these proteins are concentrated in focal adhesions where integrins cluster and bind to both the extracellular matrix and cytoskeletal proteins. I. Knezevic *et al.*, *J. Biol. Chem.* 271(27):16416-16521 (1996).

For example, talin, a 235 kD vinculin and actin binding protein, binds to the  
10 cytoplasmic domains of  $\alpha$ IIb and  $\beta$ 3 in a solid phase binding assay. I. Knezevic *et al.*, *Id.* The binding of  $\alpha$  actinin, a 100 kD vinculin binding protein and actin cross-linking protein, to the cytoplasmic domain of  $\beta$ 1 and  $\beta$ 3 in solid phase binding assays has also been observed. C.A. Otey *et al.*, *J. Biol. Chem.* 268(28):21193-21197 (1993); and C. A. Otey *et al.*, *J. Cell Biol.* 111:721-729 (1990). Binding studies have demonstrated an  
15 interaction between the cytoplasmic domain of  $\beta$ 1 and tensin, a 215 kD SH2 domain containing vinculin and actin binding protein. S. Lin *et al.* *Mol. Biol. Cell* 7 Supp. 389a, Abstract 2259 (1996).

Other cytoskeletal related proteins also interact with integrins. Skelemin, a 195 kD myosin and intermediate filament binding protein, binds to the membrane proximal  
20 regions of  $\beta$ 1 and  $\beta$ 3 cytoplasmic domains. K.B. Reddy *et al.*, *Mol. Biol. Cell* 7 Supp. 385A, Abstract 2237 (1995). These authors suggested that skelemin could link myosin and intermediate filaments to  $\beta$  integrins.

Paxillin, a vinculin binding signaling protein also binds to the cytoplasmic domain of the  $\beta$ 1 integrin. M.D. Schaller *et al.*, *J. Cell Biol.* 130:1181-1187 (1995). It is not yet  
25 known whether the  $\beta$ 1-paxillin association is direct or indirect, however paxillin was postulated as being the substrate for and tyrosine phosphorylated by tyrosine kinase pp125 FAK. The actin binding protein filamin has been shown to bind to the cytoplasmic tail of the  $\beta$ 2 integrin subunit in vitro and co-immunoprecipitated and co-localized with  $\beta$ 2 integrins in vivo. C.P. Sharma *et al.*, *J. Immunol.* 154; 3461-3470 (1995).

30 A 208 kD integrin binding protein identified as being related to the myosin light chain kinase family of serine/threonine kinases has also been reported. Walker *et al.*, *Mol.*

*Biol. Cell* 7 Supp. 385A, Abstract 2235 (1995). This kinase was said to be part of a complex of proteins including  $\alpha$ -actinin and myosin, however, it was unclear whether the kinase associated directly with the cytoplasmic tails of integrins or through a complex of proteins.

Although the cytoskeletal proteins listed above have been shown to interact with cytoplasmic domains of integrin subunits with purified proteins or peptides, it is not known how these interactions occur within cells or how these interactions are regulated. Furthermore, the integrin/cytoskeletal interactions described thus far do not occur in a phosphotyrosine-dependent manner.

### C. Tyrosine Phosphorylation of the Cytoplasmic Domain of Integrin $\beta$ Subunits

Platelet aggregation induced by a number of agonists results in the phosphorylation of tyrosine residues in the  $\beta 3$  cytoplasmic tail. Law *et al.*, *J. Biol. Chem* 271:10811-10815 (1996). In some respects, the phosphorylation of both tyrosine residues was necessary for binding to certain signaling proteins, whereas other signaling proteins bound following monophosphorylation. Furthermore, adhesion to vitronectin by cells transfected with  $\alpha v \beta 3$  induces a robust tyrosine phosphorylation of the  $\beta 3$  subunit. Blystone *et al.*, *J. Biol. Chem* 271:31458-31462 (1996).

Studies have shown that the sequences of the cytoplasmic domains of  $\beta 1$ ,  $\beta 2$  and  $\beta 3$  which contain tyrosines are important for normal integrin/cytoskeletal interactions. For example, the substitution of tyrosine 747 by alanine in  $\beta 3$  transfected into CHO cells abolished  $\beta 3$ -mediated cell spreading, blocked the recruitment of  $\alpha IIb \beta 3$  to preestablished adhesion plaques, and decreased the ability of  $\alpha IIb \beta 3$  to mediate internalization of fibrinogen-coated particles. J. Ylanne *et al.*, *J. Biol. Chem.*, 270, 9550-9557, (1995).

Additional experiments reported by Ylanne *et al.*, *Id.*, showed further that substitution of alanine for tyrosine 759 decreased cell spreading and the recruitment of  $\alpha IIb \beta 3$  to adhesion plaques, while deletion of the carboxy terminal pentapeptide that contains this sequence had an even more pronounced effect on the function of the integrin. These authors concluded integrin-mediated cell spreading does not occur because the factors that are absolutely required for integrin-mediated cell spreading cannot bind either



the  $\beta 3$  truncated at residue 757 or the integrin with tyrosine 747 of  $\beta 3$  substituted by alanine.

Point mutations in homologous domains in  $\beta 1$ - and  $\beta 2$ -containing integrins also modulate function, as these mutations affect integrin-cytoskeletal interactions by  
5 reducing focal adhesions, A. A. Reszka et. al., *J. Cell Biol.* 117:1321-1330 (1992), and integrin activation, M. L. Hibbs et. al., *J. Exp. Med.* 174:1227-1238 (1991), respectively. Tyrosine kinases similarly were found to be essential in regulating the cytoskeletal attachment of  $\alpha \text{IIb}\beta 3$ . Schoenwaelder et al., *J. Biol. Chem.* 269(51):32479-32487 (1994).

Overall, the interactions between the two tyrosines and the "cell adhesion  
10 regulatory domain" or "CARD" of residues 747-762 of the  $\beta 3$  cytoplasmic domain were reported to be essential for regulation of the adhesive function of integrin  $\beta 3$ . Liu et al., *PNAS* 93:11819-11824 (1996). A 16-amino acid sequence from the CARD inhibited adhesion of HEL and ECV 304 cells to immobilized fibrinogen by competing with  
intracellular protein-protein interactions that "engage the business end" of the integrin  $\beta 3$   
15 tail. However, the identity of cytoplasmic protein(s) interacting with CARD was said to remain to be established.

#### D. Myosin

The platelet plasma membrane is coated by a lattice-like structure, known as the  
20 membrane skeleton, that is composed of short actin filaments, actin-binding protein, spectrin, vinculin and various other proteins, not all yet identified. Fox et al., *J. Biol. Chem.* 268(34):25973-25984 (1993). On the cytoplasmic side, the skeleton appears to be associated with a network of cytoplasmic actin filaments. The membrane skeleton coats the lipid bilayer and is associated with both extracellular glycoproteins and intracellular  
25 cytoskeletal elements. Fox et al. suggested that GPIIb-IIIa induces redistribution of components of the membrane skeleton and associated signaling molecules as a step in regulating integrin-induced motile events in platelets.

Myosin is a contractile protein that interacts with actin to produce contraction or movement. The term "myosin" broadly refers to a diverse superfamily, comprised of at  
30 least 11 classes, of molecular motors capable of translocating actin filaments or of translocating vesicles or other cargo on fixed actin filaments by. One characteristic of all

myosins is their ability to reversibly bind to actin and to hydrolyze MgATP. See Figure 5 and J. R. Sellers and H.V. Goodson, Protein Profile 2:1323-1339 (1995).

All types of myosin that have been purified are multimeric and appear to possess at least three functional domains- a head, neck and tail. The head or motor domain contains nucleotide and actin binding sites and is the most conserved region of the myosin superfamily. The neck domain consists of a long single alpha helical strand from the heavy chain which is stabilized by the binding of light chain subunits. The tail region, which serves to anchor myosin so that it can translocate actin, is the most diverse primary sequence of all the regions and may serve to anchor certain myosin isoforms to cell or organelle membranes. It has been suggested that myosin clustering within a cell may occur on membranes or on actin filaments themselves. Titus, *Trends in Cell Biology* 7:119 (1997). However, the precise biochemical mechanism of interaction between the myosin tail and cytoplasmic structures has not heretofore been described.

#### **E. Signal Transduction**

The involvement of the cytoplasmic domain of GP IIb-IIIa in integrin signal transduction is inferred from mutagenesis experiments. Deletion of the cytoplasmic domain of GP IIb results in a constitutively active receptor that binds fibrinogen with an affinity equivalent to the wild-type complex, implying that the cytoplasmic tail of GP IIb has a regulatory role (T. E. O'Toole, *et al.*, *Cell Regul.* 1:883-893, (1990)). Point mutations, deletions and other truncations of GP IIb-IIIa affects the ligand binding activity of GP IIb-IIIa and its signaling response (P. E. Hughes, *et al.*, *J. Biol. Chem.* 270:12411-12417, (1995); J. Ylanne, *et al.*, *J. Biol. Chem.* 270:9550-9557, (1995)).

Chimeric, transmembrane proteins containing the cytoplasmic domain of GP IIIa, but not of GP IIb, inhibit the function of GP IIb-IIIa (Y.P. Chen *et al.*, *J. Cell Biol.* 269:18307-18310, (1994)), implying that free GP IIIa cytoplasmic domains bind proteins within cells which are necessary for normal GP IIb-IIIa function. Several proteins have been shown to bind either the transmembrane domains or the cytoplasmic domains of GP IIb or GP IIIa.

CD-9, a member of the tetraspanin family of proteins (F. Lanza, *et al.*, *J. Biol. Chem.* 266:10638-10645, 1991), has been found to interact with GP IIb-IIIa on aggregated

platelets.  $\beta 3$ -endonexin, a protein identified through two hybrid screening using the cytoplasmic domain of GP IIIa as the "bait", has been found to interact directly and selectively with the cytoplasmic tail of GP IIIa (S. Shattil *et al.*, *J. Cell. Biol.* 131:807-816, (1995)).  $\beta 3$ -endonexin shows decreased binding to the GP IIIa cytoplasmic domain containing the thrombasthenic S752-P mutation. It is not yet known whether either of these GP IIIa-binding proteins are involved in signal transduction.

Cytoplasmic proteins that bind to  $\alpha V\beta 3$  have also been described which may be interacting with the integrin at the GP IIIa cytoplasmic domain sequence. Bartfeld and coworkers (N. S. Bartfeld *et al.*, *J. Biol. Chem.* 268:17270-17276, (1993)) used immunoprecipitation from detergent lysates to show that a MW=190-kDa protein associates with the  $\alpha V\beta 3$  integrin from PDGF-stimulated 3T3 cells. IRS-1 was found to bind to the  $\alpha V\beta 3$  integrin following insulin stimulation of Rat-1 cells stably transfected with DNA encoding the human insulin receptor (K. Vuori and E. Ruoslahti, *Sci.* 266:1576-1578, (1994)). Kolanus *et al.* (*Cell* 86:233-242, (1996)) recently identified Cytohesin-1. Cytohesin-1 specifically binds to the intracellular portion of the integrin  $\beta 2$  chain, and overexpression of cytohesin-1 induces  $\beta 2$  integrin-dependent binding of Jurkat cells to ICAM-1. A novel serine/threonine kinase, ILK-1, was found to associate with the  $\beta 1$  cytoplasmic domain (Hannigan *et al.*, *Nature* 379:91-96, (1996)). Overexpression of ILK-1 inhibits adhesion to the integrin ligands fibronectin, laminin, and vitronectin.

Integrin binding to adhesive proteins and integrin signal transduction have a wide variety of physiological roles, as identified above. Enhanced signaling through integrins allows for increased cell adhesion and activation of intracellular signaling molecules which causes enhanced cell mobility and growth, enhanced cell responsiveness, and modulations in morphological transformations. Although integrins responsible for cellular function have been described and signaling events are beginning to be elucidated, the mechanism by which integrins transduce signals remains to be determined. To understand the molecular mechanisms of the inside-out and outside-in signaling roles mediated by the cytoplasmic tails of  $\beta 3$  integrin requires the identification of the intracellular molecules that interact with the intracellular tails of integrin. It has been reported that  $\alpha$ -actinin binds to  $\beta 1$  tails *in vitro* (Otey *et al.* *J. Biol. Chem.* 268:21193-21197, (1993)) but the functional relevance of these bindings is not clear. By using yeast two-hybrid, ILK-1 was identified

as a  $\beta 1$  interacting protein but ILK-1 does not bind to  $\beta 3$  (Hannigan *et al.*, *Nature* 379:91-96 (1996)).

#### F. Homologous Recombination

5 Genes can be introduced in a site directed fashion using homologous recombination. This can be used in the creation of a transgenic animal, wherein the animal would be mutated, and the phenotype of the mutation could be studied for purposes of drug screening, investigating physiologic processes, developing new products and the like. Papers discussing homologous recombination are discussed in R. Kucherlapati *et al.*,  
10 (1995) U.S. Patent No. 5,413,923.

Homologous recombination permits site-specific modifications in endogenous genes and thus inherited or acquired mutations may be corrected, and/or novel alterations may be engineered into the genome. The application of homologous recombination to gene therapy depends on the ability to carry out homologous recombination or gene  
15 targeting in normal, somatic cells for transplantation.

To prepare cells for homologous recombination, embryonic stem cells or a stem cell line may be obtained. Cells other than embryonic stem cells can be utilized (*e.g.* hematopoietic stem cells etc.) (See for more examples, J.G. Seidman *et al.*, (1994) U.S. Patent No. 5,589,369). The cells may be grown on an appropriate fibroblast fetal layer or  
20 grown in the presence of leukemia inhibiting factor (LIF) and then used. The embryonic stem cells may be injected into a blastocyst, that has been previously obtained, to provide a chimeric animal. The main advantage of the embryonic stem cell technique is that the cells transfected with the "transgene" can be tested prior to reimplantation into a female animal for gestation for integration and the effect of the transgenes. In contrast to the  
25 conventional microinjection technique, the homologous respective endogenous gene can be removed from a chromosome by homologous recombination with the transgene. By subsequent cross-breeding experiments, animals can be bred which carry the transgene on both chromosomes. If mutations are incorporated into the transgenes which block expression of the normal gene production, the endogenous genes can be eliminated by this  
30 technique and functional studies can thus be performed.

Homologous recombination can also proceed extrachromasomally, which may be of benefit when handling large gene sequences (*e.g.*, larger than 50 kb). Methods of performing extrachromosomal homologous recombination are described in R.M Kay *et al.*, (1998) U.S. Patent No. 5,721,367.

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#### **G. Production of Transgenic Animals**

Transgenic animals are genetically modified animals into which cloned genetic material has been experimentally transferred. The cloned genetic material is often referred to as a transgene. The nucleic acid sequence of the transgene is integrated at a locus of a genome where that particular nucleic acid sequence is not otherwise normally found. The transgene may consist of nucleic acid sequences derived from the genome of the same species or of a different species than the species of the target animal.

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The development of transgenic technology allows investigators to create mammals of virtually any genotype and to assess the consequences of introducing specific foreign nucleic acid sequences on the physiological and morphological characteristics of the transformed animals. The availability of transgenic animals permits cellular processes to be influenced and examined in a systematic and specific manner not achievable with most other test systems. For example, the development of transgenic animals provides biological and medical scientists with models that are useful in the study of disease. Such animals are also useful for the testing and development of new pharmaceutically active substances.

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Transgenic animals can be produced by a variety of different methods including transfection, electroporation, microinjection, gene targeting in embryonic stem cells and recombinant viral and retroviral infection (*see, e.g.*, U.S. Patent No. 4,736,866; U.S. Patent No. 5,602,307; Mullins *et al.*, *Hypertension* 22(4):630-633 (1993); Brenin *et al.*, *Surg. Oncol.* 6(2):99-110 (1997); Tuan (ed.), *Recombinant Gene Expression Protocols*, Methods in Molecular Biology No. 62, Humana Press (1997)). The term "knock-out" generally refers to mutant organisms, usually mice, which contain a null allele of a specific gene. The term "knock-in" generally refers to mutant organisms, also usually mice, into which a gene has been inserted through homologous recombination. The knock-in gene may be a mutant form of a gene which replaces the endogenous, wild-type gene.

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A number of recombinant murines have been produced, including those which express an activated oncogene sequence (U.S. Patent No. 4,736,866); express simian SV 40 T-antigen (U.S. Patent No. 5,728,915); lack the expression of interferon regulatory factor 1 (IRF-1) (U.S. Patent No. 5,731,490); exhibit dopaminergic dysfunction (U.S. Patent No. 5,723,719); express at least one human gene which participates in blood pressure control (U.S. Patent No. 5,731,489); display greater similarity to the conditions existing in naturally occurring Alzheimer's disease (U.S. Patent No. 5,720,936); have a reduced capacity to mediate cellular adhesion (U.S. Patent No. 5,602,307); possess an bovine growth hormone gene (Clutter et al., *Genetics* 143(4):1753-1760 (1996)); and, are capable of generating a fully human antibody response (McCarthy, *The Lancet* 349(9049):405 (1997)).

While murines, especially mice and rats, remain the animals of choice for most transgenic experimentation, in some instances it is preferable or even necessary to use alternative animal species. Transgenic procedures have been successfully utilized in a variety of non-murine animals, including sheep, goats, pigs, dogs, cats, monkeys, chimpanzees, hamsters, rabbits, cows and guinea pigs (*see, e.g., Kim et al., Mol. Reprod. Dev.* 46(4):515-526 (1997); Houdebine, *Reprod. Nutr. Dev.* 35(6):609-617 (1995); Petters, *Reprod. Fertil. Dev.* 6(5):643-645 (1994); Schnieke et al., *Science* 278(5346):2130-2133 (1997); and, Amoah, *J. Animal Science* 75(2):578-585 (1997)).

The method of introduction of nucleic acid fragments into recombination competent mammalian cells can be by any method which favors co-transformation of multiple nucleic acid molecules. Detailed procedures for producing transgenic animals are readily available to one skilled in the art, including the recitations in U.S. Patent No. 5,489,743 and U.S. Patent No. 5,602,307.

## SUMMARY OF THE INVENTION

The present invention provides non-human mammals comprising altered, substituted or mutant integrin cytoplasmic  $\beta$  subunit genes (and their expression products) in which at least one of the two (or more) cytoplasmic tyrosine residues of the expression product are replaced with a non-tyrosine residue such as phenylalanine. Examples of

mammals encompassed by the present invention include sheep, goat, mouse, pig, dog, cat, monkey, chimpanzee, hamster, rat, rabbit, cow and guinea pig.

More specifically, the present invention provides non-human mammals comprising a mutant GP IIIa gene and expression product wherein one or both of the cytoplasmic tyrosine residues that are capable of phosphorylation have been replaced with a non-phosphorylatable residue such as phenylalanine.

Even more specifically, the present invention provides transgenic mice comprising a mutant GP IIIa gene wherein the two cytoplasmic tyrosine residues 747 and 759 have each been replaced with phenylalanine.

The present invention also provides platelets isolated from the blood plasma of the transgenic non-human mammals of the present invention.

The present invention also provides methods of preparing a transformed non-human mammal with a mutant integrin cytoplasmic  $\beta$  subunit gene, such as the GP IIIa gene, wherein, for example, at least one of the two tyrosine residues of the endogenous GP IIIa gene has been replaced with a non-tyrosine residue to prepare the mutant GP IIIa, wherein the methods comprise: a) introducing into embryonic stem cells a nucleic acid molecule encoding the mutant integrin cytoplasmic  $\beta$  subunit gene, such as a mutant or altered GP IIIa gene, and b) regenerating a transformed non-human mammal from the cells resulting from step a).

More specifically, the present invention provides methods of preparing a transformed non-human mammal with, for example, a mutant GP IIIa gene wherein at least one of the two tyrosine residues of the endogenous GP IIIa gene has been replaced with a non-tyrosine residue to prepare the mutant GP IIIa, said method comprising:

- a) introducing into embryonic stem cells a nucleic acid molecule encoding the mutant GP IIIa gene and a selectable marker flanked by FRT sites;
- b) identifying and selecting transformed cells;
- c) removing the selectable marker from the transformed cells selected in step b) by transient transformation with FLP recombinase;
- d) injecting the transformed cells from step c) into blastocysts;

- e) regenerating a transformed non-human mammal from the blastocysts of step d), wherein the regenerated transformed non-human mammal is chimeric for the mutant GP IIIa gene;
- f) breeding the chimeric non-human mammal with a wild-type non-human mammal to produce a non-human mammal heterozygotic for the mutant GP IIIa gene;
- g) crossing a heterozygotic non-human mammal produced in step f) with a second heterozygotic non-human mammal produced in step f) ; and,
- h) selecting a non-human mammal homozygotic for the mutant GP IIIa gene from the resulting progeny.

The present invention also provides methods of comparing a characteristic between two mammals of the same species, or strain, wherein one mammal has, for example, a wild-type GP IIIa gene and the other mammal has an altered or mutant GP IIIa gene, wherein at least one of the two tyrosine residues of the wild-type GP IIIa gene has been replaced with a non-tyrosine residue in the mutant GP IIIa gene. More specifically, the present invention includes such methods using transgenic mammals wherein both cytoplasmic tyrosine residues of GP IIIa have been replaced with a non-tyrosine residue such as phenylalanine. Even more specifically, the present invention includes such methods using transgenic mammals wherein the cytoplasmic tyrosine residues 747 and 759 have both been replaced with a non-tyrosine residue such as phenylalanine.

Examples of characteristics which can be compared use in the transgenic mammals of the present invention include comparing various physiological or biological functions and responses that are mediated, in whole or in part, by the cytoplasmic  $\beta$  subunit gene of various integrin molecules. For example, a comparison of such functions would include observations and comparisons of the bleeding or clotting time between the two mammal types (i.e., between wild-type and genetically engineered animals); comparing various thrombotic responses between the two mammal types; comparing the level, extent and rate of angiogenesis between the two mammal types in response to various angiogenic stimuli;



comparing the rate and extent of tumor metastasis between the two mammal types; and comparing scope, rate and extent of inflammation between the two mammal types.

The present invention also provides methods of determining the effect of various agents on selected biological characteristics of a genetically engineered mammal  
5 expressing an altered or mutant integrin  $\beta$  subunit gene that is attributable to the expression of the GP IIIa gene, wherein the methods comprise: a) administering said agent to the transgenic mammal; b) maintaining said mammal for a desired period of time after said administration; and, c) determining whether a characteristic of said mammal that is attributable to the expression of the mutant GP IIIa gene has been affected by the  
10 administration of said agent.

Further objects and advantages of the present invention will be clear from the description and examples which follow. For example, even though the examples are directed to transgenic animals comprising mutant  $\beta 3$  subunits, the present invention can be practiced with other integrin  $\beta$  subunits that have one or more phosphorylatable tyrosine  
15 residues in the endogenous gene coding for that particular integrin  $\beta$  subunit gene.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the pairing of  $\alpha$  and  $\beta$  integrin subunits.

Figure 2 shows the cytoplasmic domain of various integrin subunits.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this  
25 invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

### I. General Description

The following discussion presents a general description of the invention as well as definitions for certain terms used herein. Portions of the invention described in this  
30

application were described by David R. Phillips at the American Heart Association meeting in San Francisco, California on April 15, 1998. The content of that presentation and any printed material related thereto are herein incorporated by reference in their entirety.

5 Previous work by the present inventors indicated that the tyrosine phosphorylation of the GP IIIa subunit of the platelet integrin GP IIb-IIIa (also known as  $\alpha$ IIb $\beta$ 3) could modulate signal transduction by this integrin (see, e.g., U.S. Patent Application No. 08/975,653, filed November 21, 1997, which is incorporated by reference herein). That GP IIIa tyrosine phosphorylation was dependent upon platelet aggregation, and the fact  
10 that signaling proteins as well as cytoskeletal proteins became associated with the phosphorylated GP IIIa, suggested to us that GP IIIa phosphorylation was important in outside-in GP IIb-IIIa signaling in platelets. Successful GP IIb-IIIa-mediated outside-in signaling is required for platelet functions such as the formation of stable platelet aggregates, an important process for normal hemostasis and one which, under aberrant  
15 conditions, can lead to the formation of occlusive thrombi. Thus, if tyrosine phosphorylation of GP IIIa is indeed a critical step in outside-in GP IIb-IIIa signaling, then it could be hypothesized that compounds capable of inhibiting such a phosphorylation event would have anti-thrombotic properties. To address the importance of tyrosine phosphorylation of GP IIIa in platelet function a mutant mouse has been generated in  
20 which the endogenous GP IIIa gene was replaced with one in which the two cytoplasmic tyrosine residues were mutated to phenylalanines (GP IIIa (Y747F, Y759F)). The platelets from such animals express GP IIIa that can not undergo tyrosine phosphorylation and therefore provide a critical tool for assessing the importance of the phosphorylation reaction for platelet function.

25 Although assessing the effect of GP IIIa mutations in transfected cell lines (i.e., *in vitro*) is useful in determining the role of various residues in GP IIIa function, the importance of the GP IIIa cytoplasmic tyrosine residues in platelet function can best be determined *in vivo*. Thus we undertook a gene replacement project in which the endogenous murine GP IIIa gene was replaced by a mutant genes with at least one non-  
30 tyrosine substitution at one of the two cytoplasmic tyrosine residues. More specifically, the endogenous murine GP IIIa gene was replaced by a mutant gene with double tyrosine

to phenylalanine substitutions in the cytoplasmic domain at positions 747 and 759 respectively. Since the murine GP IIIa gene has not been sequenced, P1 clones containing murine genomic GP IIIa were obtained using PCR-based technology with PCR primers based on the sequence of the human GP IIIa gene. DNA corresponding to the area of interest, *i.e.*, the two exons encoding the GP IIIa cytoplasmic domain, was mapped using common molecular biology techniques. The two tyrosines in the cytoplasmic domain (Y747 and Y759) were mutated to phenylalanines and a targeting vector was constructed which included the mutated GP IIIa genomic DNA as well as a selectable drug marker (neomycin resistance) flanked by FRT sites, the recognition sequences for the FLP recombinase. This vector was transfected into embryonic stem cells and cells which had undergone a homologous recombination event were identified by Southern blotting and by PCR using specific primers. Those ES cells that contained one normal GP IIIa allele and one GP IIIa (Y747F, Y759F) mutant allele as well as the neomycin resistance DNA were then transiently transfected with the FLP recombinase to remove the drug resistance DNA. Again, ES cells that had undergone the desired event were identified by both Southern blotting and PCR. The now standard protocols for the generation of mutant mice were employed. Basically, the desired mutant ES cells being injected into blastocysts in order to generate chimeric mice which were bred to wild-type mice to produce heterozygote animals expressing one normal and one mutant GP IIIa allele (as assessed by Southern blotting of tail genomic DNA and PCR). These heterozygote animals were then crossed to generate litters in which ~1/4 of the animals were homozygote for mutant GP IIIa (*ie.* the only GP IIIa expressed by these mice contained the double tyrosine to phenylalanine mutation).

These transgenic mice provide a critical tool for assessing the importance of the GP IIIa cytoplasmic tyrosine residues on platelet function. Previous work indicated that phosphorylation of the GP IIIa tyrosine residues was important in outside-in GP IIb-IIIa signaling. The platelets from the mutant mice express a GP IIb-IIIa in which the GP IIIa can not be tyrosine phosphorylated. Thus platelets from these mice can be used in a number of assays to assess the role of outside-in GP IIb-IIIa signaling on platelet function. For example, bleeding time in mutant mice will assess whether tyrosine phosphorylation of GP IIIa is critical for normal hemostasis. Also if GP IIIa tyrosine phosphorylation is an

obligatory part of outside-in signaling, one would predict the mutant mice to be defective in thrombotic responses, where the formation of very large platelet aggregates is required. We can now employ murine thrombotic models with these mutant mice to directly assess this issue. Indeed, this mutant mouse will provide information on the utility of generating  
5 therapeutics designed to interfere either directly with GP IIIa tyrosine phosphorylation, or with an event dependent on this phosphorylation e.g. interaction of phospho-GP IIIa with an adaptor protein.

Expression of the GP IIIa ( $\beta_3$ ) integrin subunit is not restricted to platelets. In endothelial cells this protein pairs with the  $\alpha_v$  subunit to form the  $\alpha_v\beta_3$  integrin. This  
10 integrin appears to play an important role in angiogenesis and tumor metastasis. In neutrophils,  $\beta_3$  is a subunit of the LRI (leucocyte responsive integrin), and is thought to be involved in inflammatory responses. Since all of the  $\beta_3$  in the mutant mouse contains the tyrosine to phenylalanine mutations in the cytoplasmic domain, this mouse can be used to examine the effect of these mutations on  $\alpha_v\beta_3$  and LRI function. Again, such analyses  
15 will provide information on whether or not inhibition of  $\beta_3$  phosphorylation will have effects on angiogenesis, tumor metastasis and/or inflammation, and thus will be of significant clinical import.

The foregoing detailed description has been given for clearness of understanding only and no unnecessary limitations should be understood therefrom as modifications will  
20 be obvious to those skilled in the art.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present  
25 disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims.

## II. Specific Embodiments

30 The presence of phosphotyrosine binding motifs within the cytoplasmic domain of  $\beta_3$  suggests that tyrosine phosphorylation of this integrin tail facilitates the recruitment of

phosphotyrosine-binding signaling proteins *Shc* and *Grb2* to the cell membrane. See Philips *et al.* (1996). The present inventors have discovered that tyrosine phosphorylation and dephosphorylation of the  $\beta 3$  integrin tail may, in an analogous manner, regulate integrin association with the cytoskeleton (see, e.g., U.S. Patent Application No. 08/975,653, filed November 21, 1997, which is incorporated by reference herein). Figure 2 provides the amino acid sequences of various human integrin  $\beta$  subunits, showing the locations of the tyrosine residues. As mentioned previously, other studies have shown that the sequences of the cytoplasmic domains of  $\beta 1$ ,  $\beta 2$  and  $\beta 3$  (GP IIIa) which contain tyrosines are important for normal functioning of GPIIb-IIIa and of other integrins. Thus, transgenic animals that express altered and mutant cytoplasmic domains of those or other integrin  $\beta$  subunits that natively contain phosphorylatable tyrosine residues are expressly included in the scope of this invention.

Also expressly contemplated are altered, modified or mutated integrin subunits that contain conservative substitutions for the phosphorylatable tyrosine residues. As used herein, a conservative amino acid substitution refers to alterations of particular residues (specifically the phosphorylatable tyrosine residues) or other alterations in the amino acid sequence that, except for the inability to undergo phosphorylation at the site of the one or more relevant cytoplasmic tyrosine residues, do not otherwise adversely affect the ability of the integrin to participate in signaling activities or to bind to one or more particular binding partners, such as myosin, for example. A substitution, insertion or deletion is said to adversely affect the normal functioning of the corresponding wild-type integrin  $\beta$  subunit peptide when the altered sequence significantly inhibits the peptide from participating in signalling activities or from associating with its native binding partners, such as myosin. For example, it is contemplated that the overall charge, structure or hydrophobic/hydrophilic properties of the  $\beta$  subunit peptide can be altered without adversely affecting activity of the peptide. Accordingly, it is contemplated that the amino acid sequence of the  $\beta$  subunit peptide can be altered, for example to render the peptide more hydrophobic or hydrophilic, to the extent that this does not adversely affecting the ability of the peptide to otherwise participate in its normal signalling

activities or to become associated with normal binding partners, such as myosin. Thus, specifically contemplated are mutant GPIIIa subunits which are

For example, in one application, altered  $\beta$  subunit peptides can be generated using standard knock-out procedures to modify any one or all of the phosphorylatable tyrosine residues. This can be accomplished using a variety of art-known procedures such as targeted recombination. Once generated, the genetically-engineered animal can be used to 1) identify biological and pathological processes mediated by the  $\beta$  subunit peptide; 2) identify proteins and other genes that interact with the  $\beta$  subunit peptide; 3) identify agents that can be exogenously supplied to overcome the absence or reduction in phosphorylation of the  $\beta$  subunit peptide; and 4) serve as an appropriate screen for identifying agents that modulate (*i.e.*, increase or decrease) the activity of the altered  $\beta$  subunit peptides.

As discussed herein, the present invention provides transgenic animals that contain non-tyrosine residues at sites where phosphorylatable tyrosine residues occur normally in endogenous  $\beta$  subunit genes. Since the non-tyrosine substitutions will not be phosphorylated as in the case where the residues are tyrosine and since normal platelet aggregation is dependent on phosphorylation occurring, the transgenic mammals of the present invention will display non-normal platelet aggregation. By comparing the physiological and morphological characteristics between the transformed and non-transformed animals one skilled in the art can thereby determine the effect of the presence or absence of cytoplasmic tyrosine phosphorylation in the GP IIIa gene on the corresponding animal.

Furthermore, the transgenic animals of the present invention can be used to identify agents that modulate (*i.e.*, either promote or further inhibit) platelet aggregation or other effects that are mediated by integrin signaling pathways. The evaluation of such agents can be conducted either *in vitro*, *in situ*, or *in vivo* by techniques known to those skilled in the art. The cells, platelets, tissues and whole organisms of the disclosed transgenic animals have utility in testing the effect of various agents for their ability to reduce or increase the processes involved with integrin-mediated cytoskeletal association. Agents which can be tested include various anticoagulant, thrombolytic and antiplatelet therapeutics and drugs. Examples of such agents include glycosaminoglycans such as

heparin; oral anticoagulants such as dicumarol, anisindione, and bromadkiolone; tissue plasminogen activator (t-PA); urokinase; aspirin; dipyridamole; and ticlopidine. See, Majerus, et al., *Anticoagulant, Thrombolytic, and Antiplatelet Drugs*, in Goodman & Gilman's *The Pharmacological Basis of Therapeutics*, Ninth Edition, Chapter 54 (1996)

5 for a more complete list of such agents and their pharmacology.

The cells and whole organisms of the transgenic animals of the present invention, quite apart from their uses in veterinary and human medicine, may also be used to investigate gene regulation, expression and organization in animals. By studying the various effects of altered or mutant integrin  $\beta$  subunits, for example the GP IIIa gene, on  
10 other cells, tissues and biological processes, one skilled in the art can ascertain the role of tyrosine phosphorylation in the normal metabolic pathways.

For further examples of diagnostic and research uses of transgenic mammals, especially transgenic mice, see U.S. Patent No. 5,569,824.

Without further description, it is believed that one of ordinary skill in the art can,  
15 using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following working examples therefore, specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

20 Other generic configurations will be apparent to one skilled in the art.

### Example 1

#### **Obtaining murine genomic GP IIIa DNA**

The sequence for the murine genomic DNA is not known and has not been published,  
25 however part of the amino acid sequence of mouse GP IIIa was available (Cietat et al. (1993) *Biochem et Biophys Res Comm.* 193: 771-778, and Dr Jean-Phillipe Rosa, *Unite INSERM* 348, Paris) and its similarity to human GP IIIa sequence suggested the genomic GP IIIa from humans and mice could be fairly similar. Therefore, several PCR primers were generated towards the mouse GP IIIa sequence in areas which, in the case of human GP IIIa, spanned the  
30 two exons known to encode the cytoplasmic domain of GP IIIa ie. exons M and N (Lanza, F. et al. (1990) *J. Biol. Chem.* 265: 18098-18103). These primers were then tested with total

murine genomic DNA and one set generated a specific band of approximately 1.5kB in the PCR (polymerase chain reaction). This band was close in size to the predicted human fragment (~1.2kB) which spanned the 3' part of exon M, the 5' part of exon N, and the intron between these two exons. The primers encoded: 1) 5' primer - residues 1834-1850 and 2) 3' primer - residues 2026-2039 of murine GP IIIa. These specific primers were then used to obtain a P1 clone containing a large fragment of murine genomic DNA which gave the same band when used as a template in PCR. Thus, we had obtained a P1 clone that contained murine genomic GP IIIa, and specifically contained the regions encoding the cytoplasmic domain of GP IIIa. Next, ~10kB of this P1 clone was mapped by a mixture of restriction digests, Southern Blotting and amino acid sequencing. It was confirmed that this was murine GP IIIa and that, similar to human GP IIIa, two exons (termed M and N after the human nomenclature) encoded the murine GP IIIa cytoplasmic region. Indeed the cytoplasmic domain amino acid residues turned out to be identical between human and mouse.

## Example 2

### Mutation of tyrosine residues 747 and 759 to phenylalanines

A .3kB PstI/SacI fragment containing exon N (which encodes the two tyrosine residues) was subcloned into pBSKS II and the two tyrosine residues were mutated to phenylalanines using standard site-directed mutagenesis techniques. Mutations were confirmed by sequencing and were also designed to introduce two new enzyme sites, a DraI site (at Y747F) and a TaqI site (at Y759F) which could be used in later identification of the mutant DNA. This mutant fragment was used to replace the wild-type PstI/SacI fragment in the murine genomic GP IIIa clone.

## Example 3

### Construction of targeting vector

An approximately 2.5kB EcoRI/HindIII fragment of murine GP IIIa, containing exon M as well as intronic genomic DNA was subcloned, using HindIII/EcoRI adaptors, into an EcoRI site of a pBS vector containing a neomycin resistant cassette. The neo<sup>r</sup> DNA was flanked by FRT recognition sequences which are sites recognized by the Flp recombinase that



can be used to excise the neo<sup>r</sup> DNA at a later stage (Dymecki S. M. (1996) *PNAS USA* 93: 6191-6196). A ~4kB HindIII fragment of GP IIIa containing exon N with the tyrosine to phenylalanine mutations was subcloned into a HindIII site on this vector to give a construct basically consisting of the mutated GP IIIa with a neo<sup>r</sup> cassette in the intron between exons M and N.

#### Example 4

##### Generation of targeted embryonic stem (ES) cells

The above targeting construct was transfected into ES cells which were then selected for neo<sup>r</sup> using the drug G418 as described in Johnson, R and Killeen, N. P (*In Gene Probes 2: a practical approach*, Eds. B. D. Hanes and S. J. Higgins. Oxford University Press p. 313-327 (1995)). ES cell which had undergone a successful homologous recombination event were identified by Southern blotting and PCR techniques. These cells now had one wild type GP IIIa allele and one containing the neo<sup>r</sup> cassette and the tyrosine to phenylalanine mutations. The drug cassette is 1.9kB in size and although it was present in an intron we wished to remove it to make sure that its presence would have no detrimental effect in our experiments. To this effect the selected ES cells were transiently transfected with the Flp recombinase which recognized the FRT sites and led to the excision of the DNA encoding the drug resistance. Thus, instead of a 1.9kB piece of extraneous DNA being present in the intron a small piece encoding the 34bp FRT recognition site (and a new XbaI site) is all that remains (Dymecki, S. M., *PNAS* 93: 6191-6196 (1996)). Again, ES cells that had undergone a successful event were selected for using Southern blotting and PCR techniques.

#### Example 5

##### Generation of mutant mice

To produce mutant mice a mutant ES cell clone (originally from a strain 129 mouse) was injected into blastocysts from a C57Bl6 mouse and these blastocysts were implanted into a pseudo-pregnant foster mother (as in Ramirez-Solis, R., Davis, A. C., and Bradley, A. *Guide to techniques in mouse development*. Methods in Enzymology 225. Eds. P. M. Wassarman, M. L. DePanphilis. Academic Press (1993) in particular p 855-878). Male chimeric animals identified by their mixed coat color (black from the C57Bl6 blastocyst and agouti from the 129

ES cells) were then mated with C57Bl6 wild-type females. The offspring were genotyped for the presence of wildtype and mutant GP IIIa alleles by Southern blotting (making use of the new XbaI site that was present only in mutant GP IIIa DNA). The offspring were a mix of wild-type and heterozygote animals and the heterozygote animals were further crossed to produce litters containing wildtype animals, heterozygote animals (ie. one wildtype and one mutant GP IIIa allele) and homozygotes (ie. both GP IIIa alleles containing the tyrosine to phenylalanine mutations).

To date the genotypes of the offspring from such matings have occurred in the expected ratio (assuming basic Mendelian genetics). Furthermore, the mutant animals are viable and express GP IIb-IIIa on their platelets at similar levels to that seen in normal animals expressing non-mutant GP IIIa when the platelets are stained with an anti-GP IIb-IIIa antibody and examined on the FACS (fluorescent activated cell sorter).

Although the present invention has been described in detail with reference to examples above, it is understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims. All cited patents, applications and publications referred to in the application are hereby incorporated by reference in their entirety.

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## WHAT IS CLAIMED

1. A non-human mammal comprising a mutant GP IIIa gene wherein at least one of the two cytoplasmic tyrosine residues encoded by the gene has been replaced with a non-tyrosine residue.

2. The non-human mammal of claim 1 wherein the non-tyrosine residue is phenylalanine.

3. The non-human mammal of claim 1 wherein the cytoplasmic tyrosine residues are tyrosine residues 747 and 759.

4. Platelets isolated from the blood plasma of the non-human mammal of claim 1.

5. The non-human mammal of claim 1 wherein the non-human mammal is selected from the group consisting of sheep, goat, mouse, pig, dog, cat, monkey, chimpanzee, hamster, rat, rabbit, cow and guinea pig.

6. The non-human mammal of claim 5 wherein the non-human mammal is a mouse.

7. The non-human mammal of claim 1 wherein both cytoplasmic tyrosine residues have been replaced with a non-tyrosine residue.

8. The non-human mammal of claim 7 wherein the non-tyrosine residues are phenylalanine.

9. The non-human mammal of claim 7 wherein the cytoplasmic tyrosine residues are tyrosine residues 747 and 759.

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10. Platelets isolated from the blood plasma of the non-human mammal of claim 7.

11. The non-human mammal of claim 7 wherein the non-human mammal is selected from the group consisting of sheep, goat, mouse, pig, dog, cat, monkey, chimpanzee, hamster, rat, rabbit, cow and guinea pig.

12. The non-human mammal of claim 11 wherein the non-human mammal is a mouse.

13. A non-human mammal expressing a transgene stably introduced into its DNA, wherein the transgene comprises DNA encoding mutant GP IIIa wherein at least one of the two cytoplasmic tyrosine residues has been replaced with a non-tyrosine residue.

14. The non-human mammal of claim 13 wherein the non-tyrosine residue is phenylalanine.

15. The non-human mammal of claim 13 wherein the cytoplasmic tyrosine residues are tyrosine residues 747 and 759.

16. Platelets isolated from the blood plasma of the non-human mammal of claim 13.

17. The non-human mammal of claim 13 wherein the non-human mammal is selected from the group consisting of sheep, goat, mouse, pig, dog, cat, monkey, chimpanzee, hamster, rat, rabbit, cow and guinea pig.

18. The non-human mammal of claim 17 wherein the non-human mammal is a mouse.

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19. The non-human mammal of claim 13 wherein both cytoplasmic tyrosine residues have been replaced with a non-tyrosine residue.

20. The non-human mammal of claim 19 wherein the non-tyrosine residues are phenylalanine.

21. The non-human mammal of claim 19 wherein the cytoplasmic tyrosine residues are tyrosine residues 747 and 759.

22. Platelets isolated from the blood plasma of the non-human mammal of claim 19.

23. The non-human mammal of claim 19 wherein the non-human mammal is selected from the group consisting of sheep, goat, mouse, pig, dog, cat, monkey, chimpanzee, hamster, rat, rabbit, cow and guinea pig.

24. The non-human mammal of claim 23 wherein the non-human mammal is a mouse.

25. A method of preparing a transformed non-human mammal with a mutant GP IIIa gene wherein at least one of the two tyrosine residues encoded by the endogenous GP IIIa gene has been replaced with a non-tyrosine residue to prepare the mutant GP IIIa, said method comprising:

- a) introducing into embryonic stem cells a nucleic acid molecule encoding the mutant GP IIIa gene;
- b) regenerating a transformed non-human mammal from the cells resulting from step a).

26. The method of claim 25 wherein the non-tyrosine residue is phenylalanine.

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27. The method of claim 25 wherein the cytoplasmic tyrosine residues are tyrosine residues 747 and 759.

28. The method of claim 25 wherein the non-human mammal is selected from the group consisting of sheep, goat, mouse, pig, dog, cat, monkey, chimpanzee, hamster, rat, rabbit, cow and guinea pig.

29. The method of claim 28 wherein the non-human mammal is a mouse.

30. The method of claim 25 wherein both cytoplasmic tyrosine residues have been replaced with a non-tyrosine residue.

31. The method of claim 30 wherein the non-tyrosine residues are phenylalanine.

32. The method of claim 30 wherein the cytoplasmic tyrosine residues are tyrosine residues 747 and 759.

33. The method of claim 30 wherein the non-human mammal is selected from the group consisting of sheep, goat, mouse, pig, dog, cat, monkey, chimpanzee, hamster, rat, rabbit, cow and guinea pig.

34. The method of claim 33 wherein the non-human mammal is a mouse.

35. The method of claim 25 further comprising breeding the transformed non-human mammal so as to produce a non-human mammal homozygotic for the mutant GP IIIa gene.

36. The method of claim 35 wherein the non-human mammal is a mouse.

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37. A method of preparing a transformed non-human mammal with a mutant GP IIIa gene wherein at least one of the two tyrosine residues encoded by endogenous GP IIIa gene has been replaced with a non-tyrosine residue to prepare the mutant GP IIIa, said method comprising:

- a) introducing into embryonic stem cells a nucleic acid molecule encoding the mutant GP IIIa gene and a selectable marker flanked by FRT sites;
- b) identifying and selecting transformed cells;
- c) removing the selectable marker from the transformed cells selected in step b) by transient transformation with FLP recombinase;
- d) injecting the transformed cells from step c) into blastocysts; and,
- e) regenerating a transformed non-human mammal from the blastocysts of step d), wherein the regenerated transformed non-human mammal is chimeric for the mutant GP IIIa gene.

38. The method of claim 37 wherein the non-tyrosine residue is phenylalanine.

39. The method of claim 37 wherein the cytoplasmic tyrosine residues are tyrosine residues 747 and 759.

40. The method of claim 37 wherein the non-human mammal is selected from the group consisting of sheep, goat, mouse, pig, dog, cat, monkey, chimpanzee, hamster, rat, rabbit, cow and guinea pig.

41. The method of claim 37 wherein the non-human mammal is a mouse.

42. The method of claim 37 wherein both cytoplasmic tyrosine residues have been replaced with a non-tyrosine residue.

43. The method of claim 37 wherein the non-tyrosine residues are phenylalanine.

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44. The method of claim 37 wherein the cytoplasmic tyrosine residues are tyrosine residues 747 and 759.

45. The method of claim 43 wherein the non-human mammal is selected from the group consisting of sheep, goat, mouse, pig, dog, cat, monkey, chimpanzee, hamster, rat, rabbit, cow and guinea pig.

46. The method of claim 45 wherein the non-human mammal is a mouse.

47. The method of claim 37 further comprising breeding the transformed non-human mammal so as to produce a non-human mammal homozygotic for the mutant GP IIIa gene.

48. The method of claim 47 wherein the non-human mammal is a mouse.

49. The method of claim 37 further comprising the following steps:

- f) breeding the chimeric non-human mammal with a wild-type non-human mammal to produce a non-human mammal heterozygotic for the mutant GP IIIa gene;
- g) crossing a heterozygotic non-human mammal produced in step f) with a second heterozygotic non-human mammal produced in step f) ; and,
- h) selecting a non-human mammal homozygotic for the mutant GP IIIa gene from the resulting progeny.

50. The method of claim 49 wherein the non-human mammal is a mouse.

51. A method of determining the importance of phosphorylation in platelet function comprising comparing a characteristic mediated by platelet function, between two mammals of the same species, wherein one mammal has a wild-type GP IIIa gene and the other mammal has a mutant GP IIIa gene, wherein at least one of the two tyrosine



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residues encoded by the wild-type GP IIIa gene has been replaced with a non-tyrosine residue encoded by the mutant GP IIIa gene.

52. The method of claim 51 wherein the non-tyrosine residue is phenylalanine.

53. The method of claim 51 wherein the cytoplasmic tyrosine residues are tyrosine residues 747 and 759.

54. The method of claim 51 wherein the non-human mammal is selected from the group consisting of sheep, goat, mouse, pig, dog, cat, monkey, chimpanzee, hamster, rat, rabbit, cow and guinea pig.

55. The method of claim 54 wherein the non-human mammal is a mouse.

56. The method of claim 51 wherein both cytoplasmic tyrosine residues have been replaced with a non-tyrosine residue.

57. The method of claim 56 wherein the non-tyrosine residues are phenylalanine.

58. The method of claim 56 wherein the cytoplasmic tyrosine residues are tyrosine residues 747 and 759.

59. The method of claim 56 wherein the non-human mammal is selected from the group consisting of sheep, goat, mouse, pig, dog, cat, monkey, chimpanzee, hamster, rat, rabbit, cow and guinea pig.

60. The method of claim 59 wherein the non-human mammal is a mouse.

61. The method of claim 51 further comprising comparing the bleeding time between the two mammal types.

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62. The method of claim 51 further comprising comparing the thrombotic responses between the two mammal types.

63. The method of claim 51 further comprising comparing angiogenesis between the two mammal types.

64. The method of claim 51 further comprising comparing tumor metastasis between the two mammal types.

65. The method of claim 51 further comprising comparing inflammation between the two mammal types.

66. The method of claim 51 wherein the mammal is a mouse.

67. A method of determining the effect of an agent on a characteristic of a mammal that is attributable to the expression of the GP IIIa gene, said method comprising;

- a) administering said agent to the mammal of claim 1;
- b) maintaining said mammal for a desired period of time after said administration; and,
- c) determining whether a characteristic of said mammal that is attributable to the expression of the mutant GP IIIa gene has been affected by the administration of said agent.

68. The method of claim 67 wherein the mammal is a mouse.

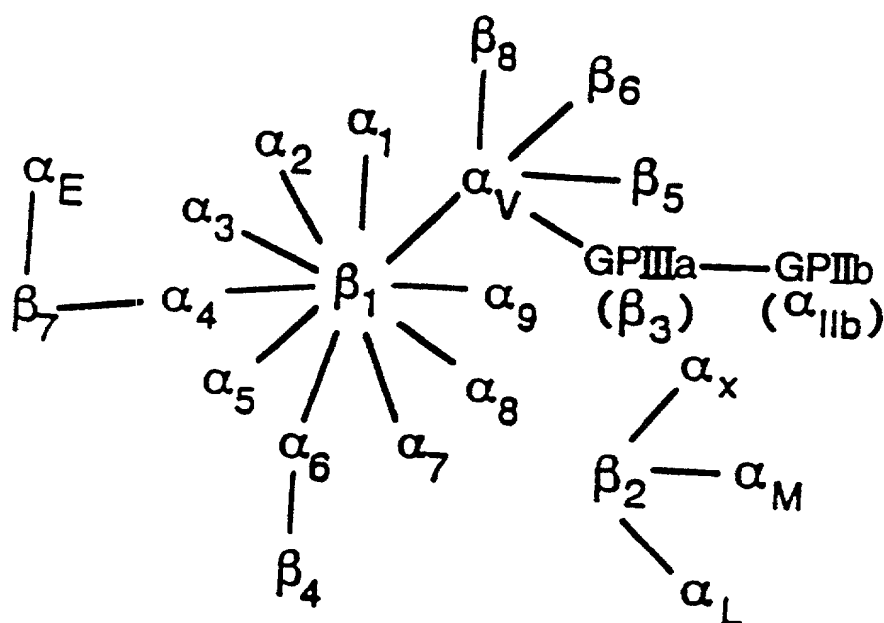


FIG. 1

GP III a ( $\beta$ 3)	KLLtIHDRK	EFAKFEeEra	rAKwtdanNP	LYKeatSift	.....Ni	tYrgt.....
$\beta$ 6	KLLVsfHDRK	EvAKFEaErs	KAKwtgtNP	LYrgststfk	.....Nv	tYkhrekqkv dIstdc
$\beta$ 1	KLLmIHDRR	EFAKEEKEkm	rAKwtdgenP	iYKSAvtIvv	.....Np	kYegk.....
$\beta$ 5	KLLVtIHDRR	EFAKFqsErs	rArYemasNP	LYrkpiStht	vdfTfnkfnK	sYngtvd...
$\beta$ 2	KaLThIsDIR	EYrrFEKEkI	ksqWnnd.NP	LFKSAIttIvm	.....Np	kFaes.....
$\beta$ 7	rLsVelyDRR	EYsrFEKEeq	qlnwkqdsNP	LYKSAItIti	.....Np	rFqeadsptI .....
Cn	KLLV-IHDRR	EFAKFE-E--	-A-W----NP	LYK-A--T--	-----N-	-Y-----

FIG. 2

# COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

U.S. DEPARTMENT OF COMMERCE  
Patent and Trademark Office

ATTORNEY DOCKET NO.: 044481-5043

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**TRANSGENIC MAMMALS EXPRESSING MUTANT GP IIIa**

the specification of which:

is attached hereto; or

was filed as United States application Serial No. \_\_\_\_\_ on \_\_\_\_\_ and was amended on \_\_\_\_\_ (if applicable); or

was filed as PCT international application Number PCT/US99/08285 on April 15, 1999 and was amended under PCT Article 19 on \_\_\_\_\_ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office information which is material to the patentability of claims presented in this application in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate or §365(a) of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

## PRIOR FOREIGN APPLICATION(S):

COUNTRY (if PCT, indicate PCT)	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
			<input type="checkbox"/> Yes <input type="checkbox"/> No
			<input type="checkbox"/> Yes <input type="checkbox"/> No
			<input type="checkbox"/> Yes <input type="checkbox"/> No
			<input type="checkbox"/> Yes <input type="checkbox"/> No

Combined Declaration For Patent Application and Power of Attorney - (Continued)  
(includes Reference to PCT International Applications)  
ATTORNEY DOCKET NO.: 044481-5043

I hereby claim the benefits under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

U.S. PROVISIONAL APPLICATIONS

U.S. PROVISIONAL APPLICATION NO.

U.S. FILING DATE

60/115,516

April 15, 1998

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or §365(c) of any PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to the patentability of claims presented in this application in accordance with Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application(s) and the national or PCT international filing date of this application:

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT:

U.S. APPLICATIONS

STATUS (Check One)

U.S. APPLICATION NO.

U.S. FILING DATE

PATENTED

PENDING

ABANDONED

**POWER OF ATTORNEY:** As a named inventor, I hereby appoint the registered practitioners of Morgan, Lewis & Bockius LLP included in the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to that Customer Number.

**Customer Number: 009629**

Direct Telephone Calls To:  
(name and telephone number)

**Reid G. Adler**  
**202-467-7501**

Combined Declaration For Patent Application and Power of Attorney - (Continued)

(includes Reference to PCT International Applications)

ATTORNEY DOCKET NO.: 044481-5043

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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[ ] Yes

[X] No